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Enemy of the (immunosuppressed) state: an update on the pathogenesis of *Aspergillus fumigatus* infection

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Summary

Aspergillus fumigatus is an opportunistic filamentous fungus that is currently the most frequent cause of invasive fungal disease in immunosuppressed individuals. Recent advances in our understanding of the pathogenesis of invasive aspergillosis have highlighted the multifactorial nature of *A. fumigatus* virulence and the complex interplay between host and microbial factors. In this review, we outline current concepts of immune recognition and evasion, angioinvasion and angiogenesis, secondary metabolism and the fungal stress response, and their respective roles in this often lethal infection.

Keywords: Aspergillus fumigatus, pathogenesis, immunosuppressed state.

Invasive fungal diseases are among the most severe complications that affect immunocompromised individuals, such as those undergoing induction chemotherapy for acute leukaemia and recipients of allogeneic haematopoietic stem cell transplants. In this high-risk population, *Aspergillus fumigatus* has emerged as the most frequent cause of invasive fungal disease. *A. fumigatus* causes invasive pulmonary aspergillosis (IPA), a syndrome whose prominent features include filamentous growth within the pulmonary parenchyma, angioinvasion, intravascular thrombosis, tissue infarction, and occasionally haematogenous dissemination (Fig 1). As with other opportunistic pathogens, the consequences of inhalation of *A. fumigatus* asexual spores (conidia) ultimately depend on the interaction between host factors (e.g. the integrity of the innate immune system) and microbial factors (virulence traits). Moreover, it has become clear that the virulence of *A. fumigatus* is multifactorial, and that the expression of specific virulence traits is often determined by the type of underlying immunosuppression of the host. Current evidence suggests that *A. fumigatus* has evolved mechanisms that aid in its survival in the environment by conferring a competitive advantage against microorganisms that share the same ecological niche. These mechanisms include thermotolerance, the secretion of extracellular proteases, nutritional scavenging, and extensive secondary metabolism. That some of these factors also aid in the survival of *A. fumigatus* in the human host may be accidental, but nonetheless contributes to the devastating outcomes associated with IPA. The clinical manifestations and treatment of aspergillosis have been recently summarized (Segal, 2009). In the present review, we discuss recent advances in our understanding of the pathogenesis of IPA, with special emphasis on the interplay between host immunity and fungal virulence factors.

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Preliminary negotiations at the mucosal interface

Aspergillus species are ubiquitous saprophytes that inhabit human environments, in particular soil and organic debris (compost). Asexual reproduction proceeds atop conidiophores, specialized structures where conidia, the infectious propagules of *Aspergillus* species, are produced and released into the air. The main function of conidia is as a means of fungal dispersion and preservation of the fungal genome in adverse environmental conditions (Osherov & May, 2001). Probably as many as a few hundred conidia are inhaled daily by humans. The conidia of *A. fumigatus* are small enough (2·0–3·5 μm in diameter) to traverse the terminal respiratory airways and reach the pulmonary alveoli, whereas the larger conidia of some other *Aspergillus* species, such as *A. flavus* and *A. niger*, tend to be deposited in the paranasal sinuses and upper airways.

Effector cells of the innate immune system are highly effective at eliminating conidia that reach the respiratory mucosa. Alveolar macrophages rapidly phagocytose conidia and destroy them within phagosomes by means of NADPH oxidase-dependent generation of reactive oxygen intermediates

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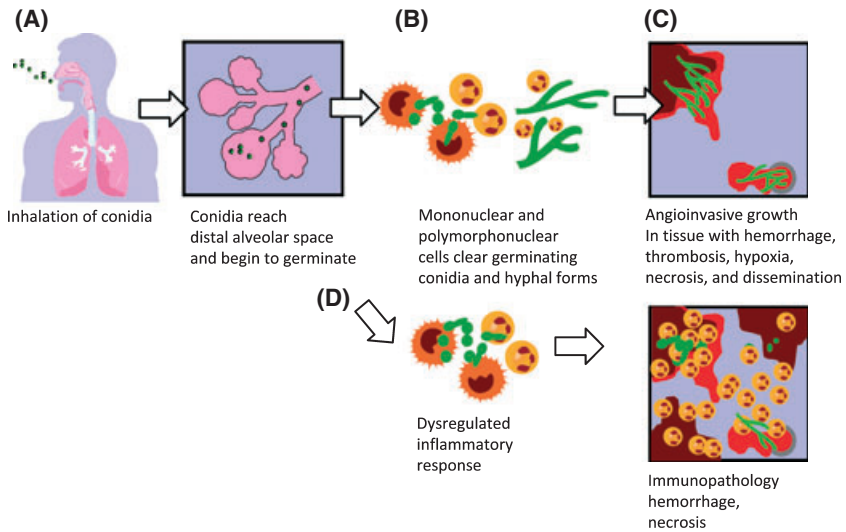

Fig 1. Pathogenesis of invasive aspergillosis in different immunological settings. (A) *A. fumigatus* conidia are inhaled by humans, and reach the terminal airways and pulmonary alveoli, owing to their small diameter (2·0–3·5 μm). (B) In the alveoli, conidia are destroyed by alveolar macrophages and polymorphonuclear leucocytes (PMNL) (see text). (C) In individuals with quantitative or qualitative defects in PMNLs, such as those who were exposed to cytotoxic drugs, *A. fumigatus* germination and tissue invasion proceed unabated. (D) Non-neutropenic hosts with a dysregulated immune response to *A. fumigatus*, for example patients receiving high dose corticosteroids, develop tissue damage as a result of PMNL recruitment, tissue infiltration and inflammatory necrosis.

(ROI) (Philippe *et al*, 2003). It has been recognized recently that polymorphonuclear leucocytes (PMNLs) also participate in defence against inhaled conidia (Bonnett *et al*, 2006). Unlike alveolar macrophages, however, PMNLs aggregate around conidia in the airways and prevent their germination in an NADPH oxidase-independent manner by releasing lactoferrin from primary granules (Bonnett *et al*, 2006; Zarember *et al*, 2007). Platelets were also found to adhere to and damage *A. fumigatus* conidia and hyphae by releasing serotonin from dense granules (Perkhofer *et al*, 2008).

In addition to the cellular components of innate immunity, a number of soluble antimicrobial molecules secreted by respiratory epithelial cells partake in resistance to *A. fumigatus* infection. Antimicrobial peptides present in respiratory secretions include surfactant proteins, lactoferrin, lysozyme and defensins. The pulmonary surfactant proteins SP-A and SP-D are collagen-containing C-type lectins (collectins) produced by type II pneumocytes. Surfactants contribute to airway mucosal resistance to infection by acting as opsonins, stimulating phagocytic cells and modulating inflammatory cytokine release (Wright, 2005). The antifungal activity of SP-D was demonstrated in immunosuppressed mice, where intranasal administration of SP-D protected animals from an otherwise fatal challenge with *A. fumigatus* conidia (Madan *et al*, 2001). Lactoferrin, an iron scavenger that is abundant in respiratory secretions (Travis *et al*, 1999), inhibits conidial growth by sequestering free iron that is required for germination (Zarember *et al*, 2007).

Conidia that escape these first lines of defence may germinate, a process that involves conidial swelling (isotropic growth) followed by the protrusion of an elongating germ tube from the conidial cell (polar growth). Conidial swelling marks the emergence of these cells from dormancy and the initiation of the asexual developmental program in *A. fumigatus* (Rhodes, 2006). Germination is triggered in warm, humid and nutrient-rich environments, conditions shared by the pulmonary alveoli and the usual ecological niche of aspergilli in compost. Polar growth occurs in tandem with nuclear division by mitosis, which triggers the formation of a septum at the base of the germ tube (Momany & Taylor, 2000). Subsequent septi are laid out at regular intervals along the elongating hyphae after each cycle of mitosis (Momany & Taylor, 2000). Filamentous growth completes the morphotypic switch from unicellular conidia to multicellular hyphae, a fungal morphotype equipped for tissue invasion.

Unlike conidia, which are effectively phagocytosed by alveolar macrophages, *A. fumigatus* hyphae are too large to be engulfed by macrophages, and are primarily targeted by PMNLs (Rex *et al*, 1990; Levitz, 2004). Swollen, but not resting conidia induce the migration of PMNLs into the lungs of experimentally infected mice (Waldorf & Diamond, 1985). These PMNLs aggregate around hyphae and damage them by the extracellular release of ROI and antimicrobial peptides from granules (Levitz *et al*, 1986; Levitz & Farrell, 1990). Moreover, dying PMNLs extrude nuclear DNA to form neutrophil extracellular traps, a highly dynamic web that is studded with fungicidal proteins and restricts hyphal growth (Bruns *et al*, 2010). The importance of PMNLs for antifungal defence can be inferred from the propensity of patients with severe prolonged neutropenia to develop IPA (Gerson *et al*, 1984).

Cloak and dagger: immune recognition and evasion

Recognition of *A. fumigatus* by innate immune cells occurs via cellular receptors known as pattern recognition receptors (PRRs), which detect pathogen-associated molecular patterns, molecular motifs common to multiple pathogenic microorganisms. The important receptor families involved in the recognition of *A. fumigatus* are the Toll-like receptors (TLRs) and lectins. The *Aspergillus* cell wall is a complex mesh of cross-linked polymers, principally β glucans, chitin and galactomannan, that forms the interface between the fungus and its environment and presents potential targets for PRR binding (Latge *et al*, 2005). Multiple sets of such ligand-receptor interactions drive the inflammatory response to *A. fumigatus*. For example, binding of conidial galactomannan by the soluble receptor pentraxin-3 (PTX3) enhances conidial uptake by alveolar macrophages and dendritic cells and results in pulmonary production of protective T helper cell type 1 (Th1) cytokines (Garlanda *et al*, 2002). The essential role of PTX3 in antifungal immunity is evident from the heightened susceptibility of PTX3-null mice to IPA (Garlanda *et al*, 2002), and from the protective effect of PTX3 against *A. fumigatus* infection in a murine bone marrow transplantation model (Gaziano *et al*, 2004). Interestingly, recent data have shown that neutrophil extracellular traps act as focal points for PTX3 activity against *A. fumigatus* conidia (Jaillon *et al*, 2007). Additional receptors that ligate galactomannan are the dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) on human dendritic cells (Serrano-Gomez *et al*, 2004) and a galactomannan-specific lectin on Langerhans cells (Persat *et al*, 2003).

TLRs and the β glucan (BG) receptor dectin-1 have complementary roles in the immune recognition of *A. fumigatus*. β-(1,3)-glucan, a major constituent of the cell wall of most pathogenic fungi, is ligated by dectin-1, a C-type lectin on PMNLs, alveolar macrophages and dendritic cells (Brown & Gordon, 2001). Recognition of BG by these innate cells triggers a robust inflammatory response that is independent of MyD88 signaling (Hohl *et al*, 2005). Importantly, it was shown that BG is displayed on the *A. fumigatus* cell wall in a stage-dependent manner (Hohl *et al*, 2005). In resting conidia, BG is concealed under an external cloak of immunologically inert hydrophobins, composed of RodA protein, which shields it from recognition by innate immune cells (Aimanianda *et al*, 2009). As conidial swelling occurs, the fungal cell wall undergoes remodeling that leads to exposure of BG polymers on the cell surface. These findings explained previous observations in which swollen and germinating conidia, but not resting conidia, triggered alveolar macrophage- and PMNL-mediated proinflammatory activity. This selective morphotype-dependent inflammatory response has been postulated to protect the host from excessive and potentially harmful inflammation. The majority of inhaled conidia pose no danger to the host and therefore do not warrant the activation of innate immunity. By contrast, germinating conidia present an imminent threat of invasive disease. Thus, BG display appears to flag those fungal elements that are most likely to be involved in tissue invasion for immune recognition.

TLR2 and TLR4 mediate the recognition of *A. fumigatus* by macrophages (Wang *et al*, 2001; Mambula *et al*, 2002; Meier *et al*, 2003; Bellocchio *et al*, 2004), although the *Aspergillus*-specific ligands for these PRRs are yet to be identified. TLR signaling is mediated through the adaptor protein MyD88 (Levitz, 2004) and culminates in activation of the transcription factor nuclear factor kB (NF-κB) and proinflammatory cytokine production. The TLR response to *A. fumigatus* is also highly morphotype-dependent: whereas both TLR2 and TLR4 are activated in response to *A. fumigatus* conidia, TLR4 signaling is lost in response to hyphae (Netea *et al*, 2003). Thus, downregulation of the TLR response to *A. fumigatus* occurs in tandem with the transition from resting conidia to the tissue invasive hyphal form, suggesting that modulation of TLR4 signaling could represent a mechanism of fungal immune evasion (Netea *et al*, 2003). Moreover, both *A. fumigatus* morphotypes were shown to have immunosuppressive effects on TLR signaling in monocytes: conidia suppress TLR2- and TLR4–induced interleukin (IL) 1β and IL6 production as a result of internalization of these receptors together with the conidia and their trafficking into phagosomes (Chai *et al*, 2009). In contrast, *A. fumigatus* hyphae selectively attenuate TLR4-mediated responses (Chai *et al*, 2009). These complex interactions may provide *A. fumigatus* with advantageous conditions that favour tissue invasion. Specifically, TLR2 activation in the absence of TLR4 signaling may drive a Th-2 type cytokine response, which impairs the host's resistance to fungal infection (Re & Strominger, 2001; Dillon *et al*, 2004). The significance of TLR signaling is further illustrated by the results of a recent study showing that IPA risk was significantly increased in stem cell transplant recipients in association with certain donor TLR4 polymorphisms (Bochud *et al*, 2008). Of note, recent studies have linked polymorphisms in additional genes encoding for components of innate immunity, such as the chemokine (C-X-C motif) ligand 10 (CXCL-10) (Mezger *et al*, 2008), the IL10 promoter (Seo *et al*, 2005; Sainz *et al*, 2007), IL1 (Sainz *et al*, 2008) and plasminogen (Zaas *et al*, 2008) with susceptibility to invasive aspergillosis in HSCT recipients, suggesting that, in the future, genetic profiling may become a powerful tool for risk stratification of susceptible patients.

Melanin synthesis has been identified as an additional mechanism involved in the survival of *A. fumigatus* conidia under the assault of alveolar macrophages and PMNLs. Conidial melanin is a polyketide polymer that forms a dense layer on the conidial surface (Jacobson, 2000). Melanin protects conidia from oxidative damage by acting as a ROI scavenger (Jahn *et al*, 1997, 2000). Accordingly, deletion of the *pksP* gene which encodes a type I polyketide synthase essential for melanin biosynthesis results in a hypovirulent mutant (Jahn *et al*, 1997; Langfelder *et al*, 1998; Tsai *et al*, 1998; Kweon *et al*, 2003).

Learn and live: the adaptive immune response to *A. fumigatus*

Activation of PRRs induces the maturation of antigen-presenting cells (primarily dendritic cells), which then prime T-cell immunity. Th1-type lymphocytes, whose signature is interferon-γ production, are necessary for effective immunity against *A. fumigatus*. A robust Th1 response enhances resistance to experimental invasive aspergillosis in mice (Cenci *et al*, 1997), whereas a Th2 response drives the development of allergic bronchopulmonary aspergillosis. Mice that survive primary infection with *A. fumigatus* become resistant to subsequent challenge with *A. fumigatus* conidia, and similar acquired resistance can be induced by administration of the soluble receptor of the Th2 cytokine IL4 (Cenci *et al*, 1997). Moreover, an increase in Th1-type cytokines *versus* Th2 cytokines is associated with improved outcomes in patients with invasive aspergillosis (Hebart *et al*, 2002). Rapid Th1 differentiation of *A. fumigatus*-specific $CD4^+$ T cells occurs during invasive aspergillosis (Rivera *et al*, 2006). This differentiation requires TLR signaling in draining lymph nodes but is TLR-independent in the airways.

Recent work has focused on the emerging role of newly recognized IL17-producing $CD4^+$ T lymphocytes in invasive aspergillosis. Th17 cells appear to promote invasive aspergillosis while exacerbating inflammatory tissue damage (Zelante *et al*, 2007). Therefore, this subset was hypothesized to induce intractable chronic aspergillosis coupled with an ineffective inflammatory response. Interestingly, researchers have recently shown that *A. fumigatus* induces a weak Th17 response as opposed to a robust Th1 response. A possible explanation lies in the ability of *A. fumigatus*, which possesses an indoleamine 2,3-dioxygenase (IDO)-family enzyme, to metabolize tryptophan into the immunoregulatory catabolite kynurenine (Chai *et al*, 2010); Kynurinine is a Th17 inhibitor and an inducer of regulatory T cells. These findings support the notion that the Th1 response is the main adaptive axis involved in resistance to *A. fumigatus* infection, while the precise role of Th17 lymphocytes remains to be defined.

Divide and conquer: angioinvasion and tissue infarction as barriers to fungal clearance

Penetration of the respiratory mucosa by *A. fumigatus* occurs both by extension of hyphae through intact epithelial cells and by germination of conidia that were endocytosed by pneumocytes. Inhaled *A. fumigatus* conidia that reach the pulmonary alveoli are readily internalized by type II pneumocytes and transported to acidic lysosomes, where most of them are destroyed (Paris *et al*, 1997; Filler & Sheppard, 2006). However, a small fraction of internalized conidia (~3%) survives within pneumocytes and serves as a latent reservoir of infection (Wasylnka & Moore, 2003). In fact, the intracellular compartment may act as a sanctuary for *A. fumigatus*, protecting it from immune surveillance. Interestingly, viable internalized *A. fumigatus* conidia inhibit the apoptosis of type II pneumocytes, suggesting that conidia survive the adverse conditions of the pulmonary alveoli by establishing an *ad hoc* symbiotic relationship with these cells (Berkova *et al*, 2006). When the internalized conidia germinate, remarkably little damage is caused to the pneumocytes.

Angioinvasion is a central feature of IPA pathogenesis. Invading hyphae traverse the alveolar-capillary barrier and penetrate endothelial cells from their abluminal side to gain access to the lumens of pulmonary arterioles. As with epithelial cells, *A. fumigatus* hyphae induce their own internalization by endothelial cells (Lopes Bezerra & Filler, 2004). This process is associated with endothelial injury, proinflammatory cytokine release, tissue factor expression on the endothelial cell surface, activation of the coagulation cascade and intravascular thrombosis (Kamai *et al*, 2006). *A. fumigatus* hyphae are potent platelet activators that promote the expression of adhesion molecules CD62P and CD63 (Rodland *et al*, 2010). Ultimately, these processes diminish the perfusion of *A. fumigatus*-infected lung parenchyma, leading to coagulative necrosis (Balloy *et al*, 2005). The typical lesion of IPA, especially in patients with defects in PMNL number or function, consists of a central zone of necrotic tissue that is heavily infected with a tangle of *A. fumigatus* hyphae and a peripheral zone of alveolar haemorrhage (Shibuya *et al*, 2004). Radiographically, this lesion corresponds to the typical macronodule surrounded by a ground-glass infiltrate (the so-called 'halo sign'), seen on computerized tomography imaging of the chest. Functionally, angioinvasion and tissue necrosis may restrict the recruitment of inflammatory cells to the site of infection, effectively sequestering *A. fumigatus*-infected tissue. Similarly, the vasculopathy associated with IPA may result in subtherapeutic pulmonary tissue drug concentrations during antifungal therapy and treatment failure despite infection with a drug-susceptible isolate (Paterson *et al*, 2003; Arendrup *et al*, 2009).

Recent work has uncovered a sterol regulatory element binding protein (SrbA) that regulates hypoxia adaptation in *A. fumigatus* (Willger *et al*, 2008). Deletion of SrbA produces a mutant strain that grows normally in atmospheric oxygen at 21% but is incapable of growing in hypoxic conditions and has abnormal hyphal branching, indicating impaired cell polarity. Moreover, the SrbA deletion mutant is unable to cause invasive disease in immunosuppressed mice (Willger *et al*, 2008). These findings suggest that the ability to grow in hypoxic conditions is an essential component of *A. fumigatus* pathogenicity.

Secondary metabolism and the multifaceted role of gliotoxin

Aspergillus species produce a remarkable number of highly diverse secondary metabolites, also known as natural products, which facilitate adaptation to diverse ecological niches (Keller *et al*, 2005). Secondary metabolites have garnered intense interest, as many of these products have beneficial pharma-

ceutical properties. However, some secondary metabolites are potent mycotoxins that damage mammalian cells. Characteristics of secondary metabolites include dispensability for fungal growth, production that is restricted to certain parts of the organism's life cycle, and aggregation of encoding genes in subtelomeric gene clusters (Keller *et al*, 2005).

Secondary metabolism was first linked with pathogenicity in *A. fumigatus* when it was discovered that deletion of LaeA, a transcription factor that broadly regulates secondary metabolism, significantly impaired virulence in a murine aspergillosis model (Bok *et al*, 2005). The LaeA deletion mutant is similar to its parental wild type strain in terms of growth, conidiation and germination rates; however, the *ΔlaeA* mutant was completely avirulent when inoculated into the airways of immunosuppressed mice (Bok *et al*, 2005). Moreover, transcriptional profiling of *A. fumigatus* at the onset of invasive infection revealed upregulation of multiple subtelomeric gene clusters ostensibly involved in secondary metabolism, relative to their expression in laboratory strains (McDonagh *et al*, 2008). Still, our understanding of the contribution of specific secondary metabolites for *A. fumigatus* virulence is incomplete. Research has focused on gliotoxin, an epipolythiodioxopiperazine immunosuppressive mycotoxin that is synthesized under the transcriptional regulation of LaeA. Gliotoxin has pleiotropic effects on various mammalian cell lines: at nanomolar concentrations it downregulates the PMNL oxidative burst by interfering with the assembly of the NADPH oxidase complex (Tsunawaki *et al*, 2004) and inhibits the activation of the redox-sensitive transcription factor NF-κB, a central regulator of inflammatory response genes in T and B lymphocytes (Pahl *et al*, 1996). At higher (micromolar) concentrations, gliotoxin induces apoptosis of peripheral blood mononuclear cells, macrophages and PMNLs (Waring *et al*, 1988; Suen *et al*, 2001; Kweon *et al*, 2003).

Importantly, transcription of the gliotoxin gene cluster is upregulated during the initiation of invasive aspergillosis (McDonagh *et al*, 2008), and gliotoxin can be detected *in vivo* in *A. fumigatus* infected hosts (Bok *et al*, 2005; Lewis *et al*, 2005). The mean concentrations of gliotoxin measured in the lungs of mice experimentally infected with *A. fumigatus* correspond with those shown to induce apoptosis (Waring *et al*, 1988). However, studies utilizing the gliotoxin nonproducing mutant *ΔgliP* revealed crosstalk between gliotoxin production, virulence, and the immunological makeup of the experimental host (Kupfahl *et al*, 2006; Sugui *et al*, 2007; Spikes *et al*, 2008). Specifically, the virulence of *ΔgliP* was attenuated in mice immunosuppressed with corticosteroids alone (a nonneutropenic model), whereas virulence in mice immunosuppressed with cyclophosphamide (neutropenic model) was comparable with that of the *gliP* wild-type strain (Kupfahl *et al*, 2006; Sugui *et al*, 2007; Spikes *et al*, 2008). These results suggested that gliotoxin's role in *A. fumigatus* pathogenicity is attributed to its action on PMNLs. Moreover, given the robust attenuation in virulence associated with LaeA deletion, it is likely that additional secondary metabolites contribute to *A. fumigatus* virulence.

An interesting aspect of gliotoxin's involvement in the pathogenesis of invasive aspergillosis is its potent antiangiogenic effect. Angiogenesis is a potentially important physiological response to the tissue hypoxia associated with angionivasive *A. fumigatus* infection. Invasion of pulmonary tissue by *A. fumigatus* activates a number of proangiogenic signaling pathways that may promote compensatory neovascularization (Fig 2). Specifically, tissue hypoxia, proinflammatory cytokines and ROI are all potent proangiogenic signals. Invasion of endothelial cells by *A. fumigatus* results in the induction of genes encoding for proinflammatory cytokines and leucocyte adhesion molecules (Kamai *et al*, 2006; Chiang *et al*, 2008). PMNLs are recruited by chemokines to the lungs, where they release hydrogen peroxide (H_2O_2) and other ROI, which upregulate NF-κB. In turn, NF-κB induces the transcription of vascular endothelial growth factor (VEGF) and other proangiogenic mediators (Huang *et al*, 2001; Maulik, 2002).

Gliotoxin exerts its antiangiogenic activity by downregulating NF-κB both directly and by reducing the concentration of H_2O_2, an important NF-κB inducer (Kroll *et al*, 1999; Choi *et al*, 2007). Gliotoxin inhibits the proteasomal degradation of the inhibitor of κBα (IκBα) thus stabilizing the NF-κB – IκBα complex and preventing nuclear translocation of NF-κB (Kroll *et al*, 1999). Moreover, as noted above, gliotoxin suppresses the PMNL oxidative burst (Tsunawaki *et al*, 2004), which may

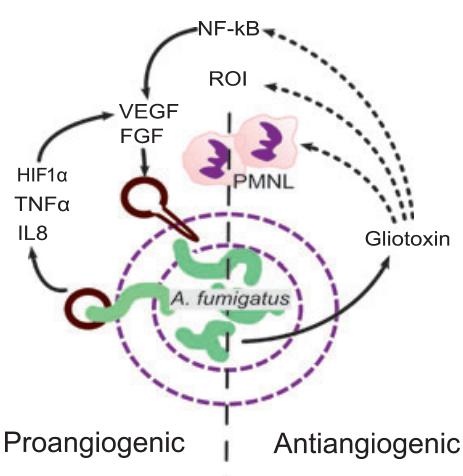

Fig 2. Pro- and antiangiogenic signals in invasive aspergillosis. Pro- and antiangiogenic signals in invasive aspergillosis are represented schematically. Circles represent infected (inner circle) and hypoperfused (outer circle) pulmonary tissue. Proangiogenic factors secreted from endothelial cells, macrophages and PMNLs appear in the left half of the figure, and antiangiogenic activities of gliotoxin secreted by *A. fumigatus* appear in the right half. TNF α: tumour necrosis factor α; HIF1α: hypoxia-inducible factor 1 α; IL8, interleukin 8; VEGF: vascular endothelial growth factor; FGF: fibroblast growth factor; ROI: reactive oxygen intermediates; NF-kB: nuclear factor κB; PMNL: polymorphonuclear leucocyte.

be an important proangiogenic signal during invasive aspergillosis. In addition, gliotoxin detoxifies secreted H_2O_2 by activating the thioredoxin redox system (Choi *et al*, 2007). The antiangiogenic activity of *A. fumigatus* secondary metabolites has been demonstrated *in vitro* as well as in animal model systems (Ben-Ami *et al*, 2009). Inhibition of angiogenesis-related gene expression was observed in cyclophosphamide/cortisone-treated mice but not mice treated with cortisone alone (Ben-Ami *et al*, 2009), consistent with the coagulative necrosis seen in neutropenic hosts with IPA (Balloy *et al*, 2005). Interestingly, gliotoxin mediates only about 40% of the antiangiogenic activity of *A. fumigatus* culture filtrates (Ben-Ami *et al*, 2009), suggesting that additional secondary metabolites are involved. For example, fumagillin is a potent antiangiognenic molecule whose activity is mediated by methionine aminopeptidase 2 inhibition (Sin *et al*, 1997). Pseurotin, another secondary metabolite with anotiangiogenic activity, has also been shown to be upregulated at the transcriptional level during the onset of invasive aspergillosis (McDonagh *et al*, 2008). Taken together, the results of these studies provide a conceptual framework of vascular pathobiology in invasive aspergillosis where neovascularization and tissue perfusion represent a net balance between the opposing effects of proangiogenic signals associated with hypoxia, proinflammatory cytokines and ROI on one side, and the antiangiogenic effects of gliotoxin on the other (Fig 2). Work is ongoing to characterize the effects of angiogenesis modulation on the outcome of invasive aspergillosis and its response to antifungal therapy.

The fungal stress response: adaptation to the human host and the potential for novel drug discovery

The host represents a hostile environment for *A. fumigatus*. Exposure to elevated temperature, high osmolality and ROI activates fungal stress response pathways, whose integrity is essential for *A. fumigatus* pathogenesis. Calcineurin is a highly conserved calcium/calmodulin regulated protein phosphatase encoded by the gene *cnaA*. Unlike the situation for *A. nidulans*, calcineurin is not essential for *A. fumigatus* survival (Steinbach *et al*, 2006), but is required for polarized hyphal growth, tissue invasion and virulence (Steinbach *et al*, 2006). Deletion of *cnaA* results in a mutant that is defective in germ tube formation, and in addition has impaired conidiation and produces conidia that lack an external hydrophobin layer; this mutant is almost avirulent in immunosuppressed mice (Steinbach *et al*, 2006). In contrast, a mutant lacking the calcineurin-dependent transcription factor CrzA grows normally but is still hypovirulent (Soriani *et al*, 2008), suggesting that calcineurin interacts with virulence in ways unrelated to its effect on polarized growth. These findings have prompted interest in calcineurin as a potential target for novel antifungal drug development. Calcineurin-inhibitors in current clinical use, such as cyclosporine A and FK506, have been shown to have inhibitory activity against *A. fumigatus* and act synergistically with antifungal drugs (Kontoyiannis *et al*, 2003; Steinbach *et al*, 2004). For example, the combination of a calcineurin inhibitor with caspofungin, both of which are normally fungistatic against *A. fumigatus*, results in fungicidal activity (Steinbach *et al*, 2004). An *in vivo* study on mice challenged with *A. fumigatus* conidia by tail vein injection and treated daily with calcineurin inhibitors showed longer survival and attenuated hyphal growth in mice treated with FK506 as compared with mice treated with cyclosporine A (High & Washburn, 1997). On a practical level, current calcineurin inhibitor formulations are unlikely to be useful as antifungal agents because of their immunosuppressive properties. Possible strategies to overcome this limitation include the discovery of novel molecules that inhibit downstream signals and lack immunosuppressive activity, or inhalational delivery of calcineurin inhibitors to the site of infection (Steinbach *et al*, 2007).

Another important stress-response signaling pathway is the cyclic AMP (cAMP)-dependent protein kinase A pathway. Adenylate cyclase, under the regulation of a G-protein-alpha-subunit (GpaB), generates cAMP which then binds to the regulatory PKA subunit (PkaR). This interaction releases the two PKA catalytic subunits PkaC1 and PkaC2, which phosphorylate downstream targets. Deletion of any of the above components of the PKA cascade attenuates virulence in mice, presumably by impairing stress adaptation in the host environment (Liebmann *et al*, 2004; Zhao *et al*, 2006). As G-protein coupled receptors are common targets for pharmaceutical manipulation, this pathway too may present opportunities for novel drug discovery (Van Dijck, 2009).

Oxidative stress, driven by the production of ROI by alveolar macrophages and PMNLs, is a major threat to fungal survival in the host. Therefore, detoxification of hydrogen peroxide by fungal catalases should theoretically enhance the pathogenicity of *A. fumigatus*. Three catalases have been identified, one specific to the conidial stage (CatA) and two produced during the mycelial stage (Cat1 and Cat2) (Paris *et al*, 2003). Additionally, the *A. fumigatus* genome contains four more putative catalase-encoding genes. However, early studies failed to show a correlation between catalase-dependent protection from peroxide stress and the pathogenicity of *A. fumigatus*. Conidia of the CatA-deficient mutant *ΔcatA*, although more susceptible to H_2O_2 *in vitro*, did not display attenuated resistance to macrophage oxidative burst or virulence. In contrast, the mutant that lacks both mycelial catalases (*Δcat1Δcat2*) had only slightly increased susceptibility to H_2O_2 but was hypovirulent in immunosuppressed rats (Paris *et al*, 2003). More recently, work done using a novel cutaneous model of invasive aspergillosis revealed distinct roles of conidial and mycelial catalases (Ben-Ami *et al*, 2010). In that model, *ΔcatA* conidia failed to germinate and initiate aspergillosis, whereas the *Δcat1Δcat2* mutant formed short stunted hyphae, indicating that conidial catalase is required for the initiation of infection and mycelial catalases are required for

Table I. Some clinically relevant questions pertaining to *Aspergillus* pathogenesis.

Question	Potential implications
What is the incubation period of invasive aspergillosis? (effects of fungal inoculum and net state of immunosuppression)	Definition of community *versus* hospital-acquired aspergillosis
What are the differences in virulence among different *Aspergillus* species or strains?	Important in risk stratification/propensity for dissemination
Do antifungals, antineoplastics or antibiotics modulate *Aspergillus* virulence?	Could influence strategies for selection of prophylaxis and treatment
Does cytokine or immunogenetic profile (e.g. TLR gene polymorphisms) provide independent means for risk stratification?	Could influence stratification strategies, intensity of monitoring and prophylaxis
What is the pathogenic role of tissue infarction in invasive aspergillosis?	Poor accessibility of immune effector cells and antifungals
What is the latency period for recovery of immune competence?	Rapidity and degree of tapering of immunosuppression (corticosteroids)
How do interactions between bacteria and *Aspergillus* on mucosal surfaces affect the pathogenesis of invasive aspergillosis?	Need for a global view on antibacterial and antifungal prophylaxis
What are the immunosuppressive effects of respiratory and systemic viral infections (e.g. influenza, RSV, CMV, and EBV)	Need to intensify monitoring and prophylaxis
How does the metabolic environment (hyperglycaemia, acidosis, osmolarity, O_2 tension, iron) modulate *Aspergillus* growth and pathogenicity?	Novel antifungal strategies; better cultivation protocols
How is the formation and integrity of granulomas regulated in subacute and chronic invasive asergillosis?	Role of TNFα inhibitors as disruptors of organized granulomas leading to disseminated infection
What are the tissue-specific differences in virulence and immune responses (e.g. cytokine/chemokine signatures)?	Dissect homeostatic responses to different patterns of tissue invasion
Regulation of mycotoxins/interface with endothelium	Enthothelial growth factors (e.g. VEGF) as adjunct compensatory strategy
Is there a fitness cost to *Aspergillus* drug resistance?	Propensity for dissemination of resistant strains
How are *Aspergillus* virulence factors differentially modulated in varying milieus of immunosuppression?	Differences in performance of *Aspergillus* diagnostics, clinical presentation, and antifungal choices
How predictive are invertebrate and mouse models of human invasive aspergillosis?	Need to develop better models of invasive aspergillosis (e.g. murine models of leukaemia or GVHD)
What is the role of biofilm formation in the pathogenesis of invasive aspergillosis?	Role in chronicity of infection, role in acquisition of invasive aspergillosis (water born transmission)

TNF, tumour necrosis factor; RSV, respiratory syncytial virus; EBV, Epstein-Barr virus; CMV, cytomegalovirus; VEGF, vascular endothelial growth factor; GVHD, graft-*versus*-host disease; TLR, Toll-like receptor.

hyphal elongation in tissue (Ben-Ami *et al*, 2010). It is unclear whether the different results obtained in these animal models reflect the subacute course of cutaneous aspergillosis relative to the more acute pulmonary model, or whether catalase production is a tissue-specific virulence factor that is not expressed during pulmonary infection.

Scavenger hunt: proteases and siderophores

Aspergillus fumigatus is well equipped for the breakdown of extracellular protein and the scavenging of nutrients. The array of proteinases produced by *A. fumigatus* includes a metalloproteinase (Ibrahim-Granet & D'Enfert, 1997), alkaline proteinase (Monod *et al*, 1993; Tang *et al*, 1993; Smith *et al*, 1994), and elastase (Kothary *et al*, 1984; Kolattukudy *et al*, 1993). These enzymes have double roles, acting both as chemical drills that clear a way for invading hyphae through host tissues, and mediating digestion and assimilation of proteinaceous substrates. However, previous attempts to link proteinase activity with pathogenicity have yielded negative results for alkaline proteinase (Tang *et al*, 1993) and inconclusive data for elastase (Kothary *et al*, 1984; Denning *et al*, 1992). More recently, two research groups reported on the cloning and deletion of *A. fumigatus* PrtT, a transcription factor that controls the expression of multiple extracellular proteases (Bergmann *et al*, 2009; Sharon *et al*, 2009). Surprisingly, although the *ΔprtT* mutant lacked extracellular protease activity, it was not attenuated in it's virulence in immunosuppressed mice. These findings suggest that *A. fumigatus* proteases under the transcriptional regulation of PrtT are indeed redundant with respect to pathogenicity, although the involvement of additional proteolytic enzymes whose expression is upregulated during pulmonary infection cannot be ruled out (Sharon *et al*, 2009).

The ability to acquire iron from the environment is common to most pathogenic organisms. Iron is a co-factor for catalases, oxygenases, and peroxidases and therefore plays an important part in resistance against oxidative stress

(Hersleth *et al*, 2006). *A. fumigatus* employs two high-affinity iron uptake systems: reductive iron uptake and siderophore-assisted iron uptake. Both systems are induced by iron deprivation, but only siderophore-assisted iron uptake appears to be essential for *Aspergillus* virulence (Schrettl *et al*, 2004). Reductive iron uptake involves reduction of iron from the ferric (Fe^{+3}) to the ferrous (Fe^{+2}) state. Ferrous iron is taken up by the FtrA/FetC complex. Siderophores, on the other hand, are low-molecular-weight chelators that bind ferric iron. *A. fumigatus* synthesizes four siderophores: two are extracellular and two are intracellular (Schrettl *et al*, 2007). The extracellular siderophores fusarinine C and triacetylfusarinine capture iron, and the intracellular siderophores ferricrocin and hydroxyferricrocin are used to store iron in hyphae and conidia respectively. Robust upregulation of siderophore gene expression is induced upon initiation of invasive aspergillosis (McDonagh *et al*, 2008). *A. fumigatus* mutants that lack any of these siderophores are significantly attenuated in virulence, indicating that both intracellular and extracellular siderophores are required for *A. fumigatus* virulence (Schrettl *et al*, 2007). Moreover, increased bone marrow iron stores were found to be an independent risk factor for invasive aspergillosis in allogeneic stem cell transplant recipients (Kontoyiannis *et al*, 2007).

Conclusions

Considerable progress has been achieved in our understanding of *A. fumigatus* pathobiology, owing in large part to the sequencing of the *A. fumigatus* genome and the utilization of animal model systems to dissect and study the various components of fungal virulence and host immune response. Future research directions in this field include elucidation of the role of angiopathology, the definition of host-specific and organ-specific *A. fumigatus* virulence factors, the study of interstrain and interspecies variations in virulence, and mapping of host genetic determinants of susceptibility to invasive aspergillosis (Table I). A more complete understanding of invasive aspergillosis is a prerequisite towards establishing effective means for the prevention and control of this often devastating infection.

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